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Multiple actions of fenamates and other nonsteroidal anti-inflammatory drugs on $GABA_A$ receptors

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Abstract

The nonsteroidal anti-inflammatory drug (NSAID) niflumic acid, a fenamate in structure, has many molecular targets, one of them being specific subtypes of the main inhibitory ligand-gated anion channel, the $GABA_A$ receptor. Here, we report on the effects of other fenamates and other classes of NSAIDs on brain picrotoxinin-sensitive $GABA_A$ receptors, using an autoradiographic assay with [^{35}S]TBPS as a ligand on mouse brain sections. We found that the other fenamates studied (flufenamic acid, meclofenamic acid, mefenamic acid and tolfenamic acid) affected the autoradiographic signal at low micromolar concentrations in a facilitatory-like allosteric fashion, i.e., without having affinity to the [^{35}S]TBPS binding site. Unlike niflumic acid that shows clear preference for inhibiting cerebellar granule cell layer $GABA_A$ receptors, the other fenamates showed little brain regional selectivity, indicating that their actions are not receptor-subtype selective. Of the non-fenamate NSAIDs studied at 100 μM concentration, diclofenac induced the greatest inhibition of the binding, which is not surprising as it has close structural similarity with the potent fenamate meclofenamic acid. Using two-electrode voltage-clamp assays on *Xenopus* oocytes, the effect of niflumic acid was found to be dependent on the β subunit variant and the presence of γ2 subunit in rat recombinant α1β and α1βγ2 $GABA_A$ receptors, with the β1 allowing the niflumic acid inhibition and β3 the stimulation of the receptor-mediated currents. In summary, the fenamate NSAIDs constitute an interesting class of compounds that could be used for development of potent $GABA_A$ receptor allosteric agonists with other targets to moderate inflammation, pain and associated anxiety/depression.

Keywords: NSAID drugs, GABA, autoradiography, niflumic acid, fenamates, recombinant $GABA_A$ receptors, Xenopus oocytes

1. Introduction

Painful conditions, such as arthritis and fibromyalgia, are comorbid with mental diseases, such as anxiety syndromes and depression (Grilli, 2017; Velly and Mohit, 2018). This interrelationship creates challenges to diagnosis and treatment options. Acute pain and inflammation are treated with nonsteroidal anti-inflammatory drugs (NSAIDs), but chronic neuropathic conditions might require drugs that have stronger actions in the central nervous system. However, some NSAIDs have been suggested to act directly e.g., on γ-aminobutyric acid type A receptors ($GABA_A$), which constitute the main fast-acting inhibitory system in the brain. $GABA_A$ receptors are targeted by acutely efficacious anxiolytic and sedative drugs, such as benzodiazepines, hypnotics and various general anaesthetics (Korpi et al., 2002; Olsen and Sieghart, 2009). While activation of selected $GABA_A$ receptor subtypes have produced analgesia in some rodent models (Knabl et al., 2008; Munro et al., 2008; Ralvenius et al., 2015), especially in chronic pain the activation of the altered "excitatory" $GABA_A$ system might worsen the condition (Coull et al., 2005; Coull et al., 2003; Kahle et al., 2013). This makes the activation of this neurotransmitter system an unattractive option to treat pain conditions with anxiety. A further problem with $GABA_A$ receptor-activating drugs is caused by a rapid occurrence of tolerance and a risk of developing dependence and addiction (Korpi et al., 2015).

It is well known that certain NSAIDs are unselective and not restricted to inhibition of cyclooxygenases (Cryer and Feldman, 1998; Vane and Botting, 2003; Vane, 2000). For instance, they also act on $GABA_A$ receptors. Especially the fenamates, mefenamic acid and niflumic acid have been reported to either stimulate or inhibit the functions of $GABA_A$ receptors in rodent model systems as well as in recombinant receptors. Previously, we reported on a strong

antagonism by niflumic acid of selected $GABA_A$ receptor subtypes (Sinkkonen et al., 2003). Mefenamic acid, flufenamic acid and niflumic acid affect $GABA_A$ receptors expressed from poly(A)+ RNA isolated from rat cerebral cortex and expressed in *Xenopus laevis* oocytes (Woodward et al., 1994), with low concentrations inducing potentiation and high concentrations inhibition. The potentiation was not blocked by flumazenil, indicating an effect not mediated by the high-affinity benzodiazepine sites. Niflumic acid had the lowest efficacy in potentiation, but the highest in inhibition, whereas mefenamic acid was strongly potentiating and weakly inhibiting. The effects of mefenamic acid on $GABA_A$ receptor function is dependent on the β subunits in a complex fashion (Halliwell et al., 1999). It facilitates the $GABA_A$ currents in recombinant α1β2 and α1β2γ3 receptors, is inactive in α1β1γ2 and inhibitory in α1β1 receptors. The activation profile was traced to the well-known single amino acid residue "N290" in the β1 subunits.

Here, we report for the first time on global effects of NSAIDs on mouse brain $GABA_A$ receptors, by studying brain regional effects of fenamates and other NSAIDs on the regulation of $GABA_A$ receptor-integral anion channels with autoradiography using the channel-binding ligand *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) with special emphasis on the GABA-insensitive atypical $GABA_A$ receptor populations as these are the only defined ones which can be easily visualized with [^{35}S]TBPS binding (Halonen et al., 2009). Furthermore, we describe in more detail the subunit-dependencies of niflumic acid actions on a number of recombinant $GABA_A$ receptor subtypes heterologously expressed in *Xenopus laevis* oocytes.

2. Materials and methods

2.1. Animals and tissues

The autoradiographic data were collected using six male 2-month-old C57BL/6NHsd mice (Harlan Netherland, Horst, The Netherlands). The mice were briefly anesthetized using CO_2 and decapitated, the brains dissected out and frozen on solid CO_2 and stored at -80°C until sectioned. The electrophysiological data were from stage V-VI oocytes obtained from female *Xenopus laevis* frogs (Horst Kähler, Hamburg, Germany), anesthetized with 0.2% tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA). Animal procedures were approved by the Southern Finland provincial government (ESAVI/686/04.10.03/2012; Eläinkoelautakunta, ELLA).

2.2. [^{35}S]TBPS autoradiography

We used 14-µm-thick horizontal cryostat sections thaw-mounted onto gelatin-coated object glasses from naive adult mice, cut with a Leica CM 3050S cryostat (Leica Microsystems, Benheim, Germany) (Korpi et al., 1992; Makela et al., 1997; Sinkkonen et al., 2001). The sections were preincubated in ice-cold buffer containing 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl for 15 min. We used two different assays. In the first one, we aimed at revealing allosteric drug sensitivities of the main GABA-sensitive $GABA_A$ receptor populations, with GABAergic agonists reducing the binding and antagonists increasing it (Korpi and Luddens, 1997). In the second one, we assessed the drug sensitivities of minor thalamus- and cerebellum-enriched atypical (GABA-insensitive, GIS) $GABA_A$ receptor populations (Halonen et al., 2009; Sinkkonen et al., 2001). The ligand *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) that we used is highly selective to $GABA_A$ receptors as compared to strychnine-sensitive glycine receptors (Rienitz et al., 1987). The sections were incubated with 6 nM [^{35}S]TBPS (Perkin-Elmer, Boston, MA, USA) in the incubation buffer (50 mM Tris-HCl, 120 mM NaCl, pH 7.4) at room temperature for 90 min, using

either full or one sixth dilution radioactivity, for the GIS and main receptor populations, respectively. This was carried out in the presence and absence of GABA and drugs. Nonspecific binding was determined with 100 µM picrotoxinin (Sigma-Aldrich). For the study of the main receptor population, the sections were washed 3 x 15 s in ice-cold 10 mM Tris-HCl and quickly desalted in ice-cold distilled H_2O. The GIS sections were washed 3 x 30 min in ice-cold 10 mM Tris-HCl and quickly desalted in ice-cold distilled H_2O. After the washing and air-drying, the sections were exposed to Biomax MR film (Eastman Kodak, Rochester, NY, USA) for 1-6 weeks with plastic ^{14}C-radioactivity standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Binding densities in selected brain regions were quantitated with MCID IAS-imaging software (Imaging Research Inc., St. Catharine's, Ontario, Canada) and standardized with radioactivity values on the basis of the simultaneously exposed standards. Nonspecific binding was subtracted from all values.

2.3. Recombinant $GABA_A$ receptor subunits and preparation of cRNAs

Capped cRNAs coding for rat $GABA_A$ receptor subunits α1, β1, β3, and γ2S (Luddens et al., 1990; Shivers et al., 1989; Ymer et al., 1989) were transcribed *in vitro* from pRK5 plasmids with Sp6 as promoter using mMessage mMachine kit (Ambion, Austin, TX, USA) according to manufacturer's instructions.

2.4. Oocyte electrophysiology of recombinant $GABA_A$ receptors

Experiments using recombinant $GABA_A$ receptors were carried out as described in (Sinkkonen et al., 2003). In brief, isolated oocytes were stored in normal frog Ringer: 115 mM NaCl, 2.5 mM KCl, 18 mM $CaCl_2$, and 10 mM HEPES, pH 7.5. Oocytes were defolliculated and

injected via a glass micropipette with 46 nl of a solution containing mixtures of subunit cRNAs (0.1–2.5 μg/μl) or pure H_2O with Drummond Nanoject injector (Drummond Scientific Co., Broomall, PA, USA). The oocytes were incubated at 19°C in incubation solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 0.5 mM theophylline, 2 mM sodium pyruvate, 10 U/ml penicillin and 10 μg/ml streptomycin, pH 7.5]. After injection (2 h–1 day) oocytes were digested for 30 min in Ca^{2+}-free medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.5) containing 0.3 U/ml collagenase type IA (Sigma-Aldrich). Thereafter, the oocytes were incubated in incubation solution until recordings. For each experiment, oocytes from at least two different frogs were used. Electrophysiological recordings were made 1 to 3 days after cRNA injection. Oocytes were perfused with normal frog Ringer + drugs at a flow rate of 1 ml/min at room temperature (22°C) using an Ismatec pump (Ismatec, Glattbrugg-Zürich, Switzerland) and 17 channel perfusion system with pinch valves.

Drug combinations were mixed before experiments. As negative control we employed furosemide which acts highly specifically on $\alpha6\beta2(\gamma2)$ and $\alpha6\beta3(\gamma2)$ receptors but not on $\alpha1$ or $\beta1$ subunit-containing receptors (Korpi et al., 1995; Korpi and Luddens, 1997). Niflumic acid at 1000 μM concentration served as positive control. The GABA concentrations used in the experiments were chosen according to the expected $EC_{75,\ GABA}$ (except for $\alpha1\beta3\gamma2$ receptors when $EC_{25,GABA}$ was used), which was in good agreement with the obtained GABA concentration-response curves for the four receptor subunit combinations (data not shown). We expected the largest effects of the analyzed compounds at this GABA concentration as the compounds were inhibitory in some receptor isoforms.

As positive control oocytes were impaled with two microelectrodes (1.0–2.5 MΩ) filled with 3 M KCl plus 10 mM EGTA, and voltage clamped at -50 mV with Turbo TEC-05 two electrode

voltage-clamp amplifier (NPI Electronic GmbH, Tamm, Germany). Experiments were controlled by Egg-Works experimental control and data acquisition software program version 3.0.2 (NPI Electronic GmbH). GABA was dissolved in NFR. Niflumic acid was dissolved in 0.1 M NaOH, stocks were diluted in NFR to a concentration of 10 mM, and pH was adjusted to 7.5. Drugs were applied for 10 s unless otherwise stated, and 180- to 600-s washout periods were used, depending on drug concentrations. In the GABA concentration-response experiments, any given GABA concentration was first applied alone and thereafter in the presence of niflumic acid.

2.5. Drugs

All fenamates and other NSAIDs were obtained from Sigma-Aldrich. They were dissolved at 1 mM stock concentration into 0.1 M NaOH, and adjusted for pH 7.5 after final dilutions, if different from that.

2.6. Data Analyses and Statistics

Data analyses were performed using EggWorks Reader version 3.0.2 (NPI Electronic GmbH) and GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA) programs.

For autoradiography, the specific [^{35}S]TBPS binding values were determined by subtracting the nonspecific binding values from the corresponding total binding values under each incubation condition. To assess the statistical significance of the drug effects on [^{35}S]TBPS binding, one-way or two-way analyses of variance (ANOVA) and Dunnett’s post hoc test were used. Student’s t test was used for fenamate effects on GABA-insensitive binding.

For electrophysiological recordings, the amplitudes of peak currents induced by GABA and drug applications were determined from recorded traces, normalized to the corresponding GABA-induced peak currents estimated linearly between the GABA peak currents closest before and after the applications of GABA with the drugs, and presented as a percentage of the control GABA current. The peak currents induced by various GABA concentrations for each oocyte were normalized by setting the maximal GABA current without niflumic acid to 100%, and the GABA concentration-response curves were generated using nonlinear regression fit to a sigmoidal concentration-response curve. The statistical significance of the niflumic acid modulation of the GABA response was assessed with one-way ANOVA and Dunnett's *post hoc* test. Furosemide and niflumic acid effects at 1,000 μM without additional GABA were assessed using Student's *t*-test.

3. Results

3.1. Effects of niflumic acid and other fenamates on $GABA_A$ receptor binding

Previously, we have reported on a strong antagonism by niflumic acid of selected $GABA_A$ receptor subtypes (Sinkkonen et al., 2003). Now we wanted to determine whether the structurally close analogues flufenamic acid, meclofenamic acid, mefenamic acid and tolfenamic acid (Fig. 1) have similar effects. Concentration-response curves for these other fenamates clearly differed from those of niflumic acid in all brain regions. Niflumic acid had no effect on forebrain [^{35}S]TBPS binding without added 3 μM GABA (Fig. 2), and only a minor inhibitory effect at the highest concentration in the presence of GABA. The other fenamates, except for flufenamic acid, robustly inhibited the binding at 10 μM concentration in both incubation conditions [one-way ANOVAs for each brain region $F(6,35) > 20$, $P < 0.0001$]. Flufenamic acid plainly differed from niflumic acid in

the presence of GABA. The brain regional efficacy by the fenamates indicates a broad $GABA_A$ receptor subtype selectivity, unlike that detected previously for niflumic acid (Sinkkonen et al., 2003) and confirmed here, as niflumic acid (10 μM) did not inhibit the [^{35}S]TBPS binding without or with added 3 μM GABA, but rather increased it [$F(6,35) = 67.5$, $P < 0.001$; $F(6,35) = 41.9$, $P < 0.001$, respectively].

A high saturating GABA concentration (1 mM) abolished most of the picrotoxinin-sensitive [^{35}S]TBPS binding in all brain regions, except for the thalamus and cerebellar granule cell layer, which retained 5-10% of the basal binding [Fig. 3, Table 1; (Sinkkonen et al., 2001)]. We also tested the effects of fenamates on this atypical GABA-insensitive binding component and found that the fenamates other than niflumic acid abolished this binding almost completely at micromolar concentrations [Fig. 3, Table 1; ANOVA $F(8, 45) = 38{,}76$, $P < 0.0001$ and $F(9, 50) = 46{,}01$, $P < 0.0001$ for the thalamus and cerebellar granule cell layer, respectively], whereas niflumic acid at 100 μM failed to affect the thalamic binding and robustly enhanced the cerebellar granule cell layer binding, in agreement with (Sinkkonen et al., 2001). The most potent fenamate appeared to be meclofenamic acid that reduced the binding to almost half in both brain regions at 1 μM concentration (Table 1).

3.2. Effects of non-fenamate nonsteroidal anti-inflammatory drugs on $GABA_A$ receptor binding

We then tested non-fenamate NSAIDs (Fig. 1) for their effects on [^{35}S]TBPS binding with or without added 3 μM GABA in various brain regions. All brain regions showed sensitivity to some of the NSAIDs [Fig. 4; 2-way ANOVAs for brain region $F(6,231) = 684.7$, $P < 0.0001$, drug $F(10,231) = 18.54$, $P < 0.0001$, and brain region x drug interaction $F(60, 231) = 1.3$, $P > 0.05$, and for brain region $F(6,231) = 2115$, $P < 0.0001$, drug $F(10,231) = 113.6$, $P < 0.0001$, and brain region x drug

interaction F(60, 231) = 10.7, P < 0.0001, for the total binding and for the binding in the presence of 3 μM GABA, respectively]. Most drugs at 100 μM concentration failed to affect basal and GABA-inhibited binding (Fig. 4), but diclofenac and MF-tricyclic clearly inhibited the binding in the presence of GABA, but had hardly any effects without it. Also rofecoxib had some inhibitory effects on the binding, but less consistently than diclofenac and MF-tricyclic.

3.3. Molecular determinants of niflumic acid action on recombinant $GABA_A$ receptors

Since niflumic acid effects are markedly different from those of other fenamates, we wanted to gain insight on which features of the $GABA_A$ receptor subunits its actions depend. To that aim we investigated niflumic acid's action on four defined $GABA_A$ receptor subtypes by measuring its effect on GABA-induced Cl^--influx into *Xenopus laevis* oocytes injected with rat α1 subunit RNA together with either the rat β1 or rat β3 subunit RNA. Additionally, we looked at the action of this fenamate on GABA-induced currents of rat α1β1γ2 and α1β3γ2 receptors.

For both receptors lacking the γ2 subunit niflumic acid dose-dependently decreased the GABA-induced current [Fig. 5A; niflumic acid effect F(4,50) = 73.2, P < 0.0001; receptor effect F(1,50) = 87.0, P < 0.0001], though niflumic acid was more potent on β1- than on β3-containing receptors by a factor >10 (Fig. 5B). When α1/β1 and α1/β3 RNA was co-injected with γ2 RNA, α1β1γ2 receptor responses to GABA were still inhibited by increasing concentrations of niflumic acid [Fig. 6 A, B; niflumic acid effect F(4,50) = 751.2, P < 0.0001; receptor effect F(1,50) = 6.9, P < 0.001]. However, niflumic acid stimulated the GABA response more than two-fold in α1β3γ2 receptors.

4. Discussion

Global, strong effects of the fenamates and some other NSAIDs on brain $GABA_A$ receptors indicate either effects on the main receptor subtypes (e.g., $\alpha1\beta2\gamma2$ receptors) or very strong effects on minor subtypes, such as $\alpha1\beta3\gamma2$ receptors. In the absence of saturating 1-mM GABA concentration, the active fenamate drugs behaved like agonists (Korpi et al., 1996; Makela et al., 1997), reducing the binding of the channel ligand [^{35}S]TBPS practically in all brain regions, except for niflumic acid in the cerebellar cortex. We have reported on the antagonist-like action of niflumic acid on $GABA_A$ receptors in the cerebellar granule cell layer (see also Figs 2-3, Table 1), which effect is dependent on $\alpha6$ subunit-containing $GABA_A$ receptors (Sinkkonen et al., 2003). Here, we found that the other studied fenamates did not recapitulate this effect, but had agonist-like effect also in the cerebellum. Furthermore, the fenamates reduced the GABA-insensitive [^{35}S]TBPS binding component, which is dependent on $\alpha1$ subunit-containing receptors in the forebrain and on $\alpha6$ containing receptors in the cerebellum (Halonen et al., 2009). Thus, our results suggest that the fenamates have the potential to enhance the function of a wide range of $GABA_A$ receptor subtypes in the brain. However, we could not establish any clear structure-activity relationship for their activity.

Of the non-fenamate NSAIDs that had agonist-like activity, the clinically widely used diclofenac was the most interesting (Fig. 4). Its structure resembles those of the fenamates (Fig. 1) and it did cause a significant reduction in the binding of [^{35}S]TBPS in most brain regions (Fig. 4B). Also the experimental MF-tricyclic and the newer cyclooxygenase-2-selective rofecoxib had some agonist-like effect, but the other drugs tested were rather inactive in this assay. Some NSAIDs have been shown to potentiate the antagonistic effects of quinoline-antibiotics on $GABA_A$ receptors. Thus, the antagonism by enoxacin, a guinolone antibiotic, on $GABA_A$ responses in frog dorsal root ganglion sensory neurons were potentiated by indomethacin and ibuprofen, but not by diclofenac,

piroxicam and paracetamol (Yakushiji et al., 1992). It should be kept in mind that fenamates [flufenamic acid as an example (Guinamard et al., 2013)] and diclofenac have multiple ion channel targets other than $GABA_A$ receptors that can contribute to clinical efficacy and adverse effects, albeit often at higher concentrations than needed to inhibit cyclooxygenases (Gwanyanya et al., 2012).

The effects of niflumic acid on recombinant $GABA_A$ receptor function was dependent on the β and γ subunits in a complex fashion. Niflumic acid had inhibitory effects on GABA-induced currents in α1β1, α1β3 and α1β1γ2 recombinant receptors, whereas the effect was positive in α1β3γ2 receptors (Fig. 5 and 6). This is partially in line with the finding that mefenamic acid facilitates the $GABA_A$ currents at recombinant α1β2 and α1β2γ3 receptors, is inactive at α1β1γ2 and inhibitory at α1β1 receptors (Halliwell et al., 1999). The activation profile was traced to a single amino acid residue "N290" in the β2/3 subunits, which appears to mediate the effects of a number of drugs on $GABA_A$ ion channels [for pharmacological listing of these compounds, see (Sieghart and Savic, 2018)]. Other fenamates have a similar profile as mefenamic acid (Smith et al., 2004). Since the β1 subunit-containing receptors have been suggested to mediate sedative drug actions more readily than β3 containing receptors, the fenamates should not be very sedative. However, the latter receptors are thought to be more involved in anaesthesia than β1 receptors (Jurd et al., 2003; Yanovsky et al., 2012).

Fenamates are also functionally active on native $GABA_A$ receptors, e.g. in cultured primary hippocampal neurons from rats (Coyne et al., 2007). Oral administration of mefenamic acid to mice results in such high brain concentrations that should be relevant for potentiating $GABA_A$ receptors (Gee et al., 2010). Indeed, mefenamic acid has been shown to be neuroprotective in adult male Wistar rats in a transient ischemic stroke model (Khansari and Halliwell, 2009). As well, in a rat pilocarpine-model of seizures, intraperitoneal mefenamic acid has been protective, while

ibuprofen and indomethacin not (Ikonomidou-Turski et al., 1988). In that study, high doses (30-40 mg/kg) of mefenamic acid induced sedation and reduced muscle tone. Mefenamic acid ip 5 mg/kg daily for 3 weeks reduced learning deficits in a rat model of Alzheimer's disease (Joo et al., 2006). Tolfenamic acid has been reported to have therapeutic effect on alcohol hangover and migraine attacks (Hakkarainen et al., 1979; Parantainen, 1983). All these results suggest that fenamates are taken up into the CNS at efficient concentrations. Though the carboxy groups provide some polarity to all fenamates studied, the calculated partition coeffcients (logP values ranged from 4.3 to 5.1) between water and oil [(Tetko et al., 2005) http://vcclab.org] do not contradict their passage of the blood brain barrier (Pajouhesh and Lenz, 2005). We are not aware of any studies on possible $GABA_A$ receptor-mediated anxiolytic effects of NSAIDs and especially those of fenamates. On the contrary, intracerebroventricular infusion in rats of niflumic acid and flufenamic acid reduced high-dose ethanol-induced loss of righting reflex as a measure of alcohol intoxication, but not low dose-induced sedation (Carter et al., 2016), which was correlated with their antagonism of calcium-activated anion channels. Thus, any agonistic effect on $GABA_A$ receptors should have potentiated the alcohol sedation.

Very recently there have been efforts to develop positron emission tomography ligands for cyclooxygenase 1 and 2. The first reports with monkeys have suggested that both COX-1 (Kim et al., 2018) and COX-2 (Kumar et al., 2018) ligands pass through the blood-brain barrier producing partially pharmacologically specific accumulation in selected brain structures.

5. Conclusions

Our results indicate robust agonist-like effect of fenamates and some other NSAIDs on the main fast-acting inhibitory neurotransmitter system, $GABA_A$ receptor, in the brain. It remains to be studied whether these actions are relevant in the effects of these drugs on pain and comorbid

neuropsychiatric symptoms, and whether these drugs could be used to develop even more potent allosteric agonists without losing the anti-inflammatory efficacy.

The fenamates and diclofenac were the compounds with the highest efficacy in most brain regions studied under basal conditions as well as in the presence of 3 µM GABA, whereas e.g. the structurally unrelated sulindac was effective only in the latter condition for $GABA_A$ receptor subpopulation in the inferior colliculus and the thalamus. Furthermore, whereas the fenamates and diclofenac further inhibited [^{35}S]TBPS binding, sulindac potentiated it, suggesting that different NSAIDs might have different effects on co-morbid symptoms in pain conditions.

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Table 1. The differential effects of niflumic acid vs. other fenamates on GABA-insensitive [^{35}S]TBPS binding to $GABA_A$ receptors in the mouse thalamus and cerebellar granule cell layer.

Brain area	GABA 1 mM	% of basal	Niflumic acid	Flufenamic acid		Meclofenamic acid		Mefenamic acid		Tolfenamic acid	
	nCi/g		100 μM	3 μM	30 μM	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM
Thalamus	38 ± 4	5 ± 1	97 ± 4	76 ± 11	11 ± 6 [a]	56 ± 4 [a]	0 [a]	114 ± 6	5.1 ± 3.2 [a]	115 ± 13	0 [a]
CbGCL	35 ± 2	11 ± 1	343 ± 33[a]	81 ± 18	23 ± 11 [a]	65 ± 8 [a]	3 ± 2 [a]	93 ± 5	20 ± 8 [a]	90 ± 6	14 ± 5 [a]

Values for the fenamates are mean percentages ± S.E.M. of the corresponding values in the presence of a saturating concentration of GABA alone (*n* = 6 mice). CbGCL, cerebellar granule cell layer. See Figure 3 for representative images.

[a] $P < 0.001$ for the significance of the difference from the 1-mM GABA-inhibited binding (ANOVA, followed by Dunnett's test).

Legends to figures

Fig. 1. Structures of the anti-inflammatory compounds and paracetamol studied for their effects on $GABA_A$ receptors with the 2-(phenylamino)benzoic acid moiety being the IUPAC name of fenamic acid in the first five compounds and diclofenac being a closely related structure.

Fig. 2. Effects of fenamates on basal and GABA-inhibited [^{35}S]TBPS binding to $GABA_A$ receptors in various mouse brain regions. (**A**) Representative images, demonstrating clear enhancement of the GABA effect on binding in all brain regions by 10 μM concentrations of the fenamates other than niflumic acid and in some areas flufenamic acid. Ctx, cerebral cortex; CbGCL, cerebellar granule cell layer; Hi, hippocampus; IC, inferior colliculus; Str, striatum; Th, thalamus. (**B**) Concentration-response curves for the effects of fenamates on basal binding. (**C**) Concentration-response curves for the effects of fenamates on 3-μM GABA-inhibited binding. The data points are means ± S.E.M. of the corresponding GABA values (*n* = 4-6).

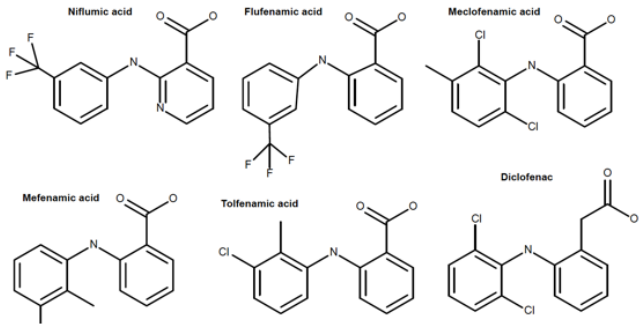
Fig. 3. The effects of fenamates on atypical GABA-insensitive binding of [^{35}S]TBPS to $GABA_A$ receptors in mouse brain sections. Picrotoxinin (Ptx) was used to define the nonspecific (NS) binding in the presence of saturating high concentration of GABA (1 mM). Nifl, niflumic acid; Mecl, meclofenamic acid; Mefe, mefenamic acid; Tolf, tofenamic acid. Flufenamic acid produced visually similar images as the fenamates other than niflumic acid, but were accidentally smeared on sections, allowing quantification of the thalamic and cerebellar granule cell layer (CbGCL) bindings (Table 1) but not imaging. The number indicate micromolar concentrations. Note that niflumic acid antagonized the effect of GABA in the CbGCL, while the other fenamates almost fully abolished the residual GABA-insensitive binding in the thalamus and CbGCL.

Fig. 4. The effects of nonsteroidal anti-inflammatory drugs on the basal and GABA-inhibited [^{35}S]TBPS binding to $GABA_A$ receptors in various mouse brain regions. (**A**) Representative images, demonstrating enhancement of the 3-μM GABA effect on binding by the positive control, fenamate meclofenamic acid (Mecl, at 1 μM, see Fig. 2BC), and by 100 μM concentrations of diclofenac (Dicl), rofecoxib (Rof) and MF-tricyclic (MF), but not by paracetamol (Par, acetaminophen), which slightly increased the binding in some brain regions in the presence of GABA. Ctx, cerebral cortex; (Cb)GCL, cerebellar granule cell layer; Hi, hippocampus; IC, inferior colliculus; (Cb)ML, cerebellar molecular layer; Str, striatum; Th, thalamus. (**B**) Regional effects of the studied NSAIDs on [^{35}S]TBPS binding in selected brain regions. Bars are means + S.E.M. (*n*=4). * $P < 0.05$, $^{\&}$ $P < 0.01$, $^{\#}$ $P < 0.001$ for the significance of the difference from the basal or GABA-inhibited binding in the absence of drugs within the brain region (ANOVA, followed by Dunnett's test).

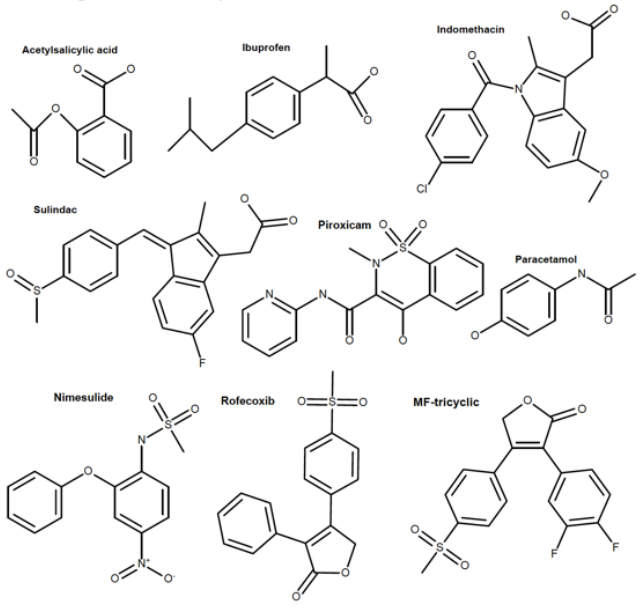
Fig. 5. (**A**) Representative current traces of niflumic acid and furosemide modulation on 10 μM and 20 μM GABA-induced currents in recombinant α1β1 and α1β3 $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. n = 7 and 5 for α1β1 and α1β3 combinations, respectively. F100, furosemide 100 μM. (**B**) Niflumic acid effects represented as mean percentage ± S.E.M., the GABA 30 μM and GABA 20 μM control responses being set to 100 %. F, furosemide 100 μM + GABA 30 μM or GABA 20 μM; N, 1000 μM niflumic acid alone. *** $P < 0.001$ for the significance of the difference from the corresponding control value (one-way ANOVA, followed by Dunnett's test for niflumic acid in the presence of GABA, Student's t-test for furosemide or niflumic acid alone). The dashed line represents the GABA control value. The EC_{50} values were 6.1 ± 1.3 μM and 10.6 ± 1.1 μM for α1β1 and α1β3, respectively.

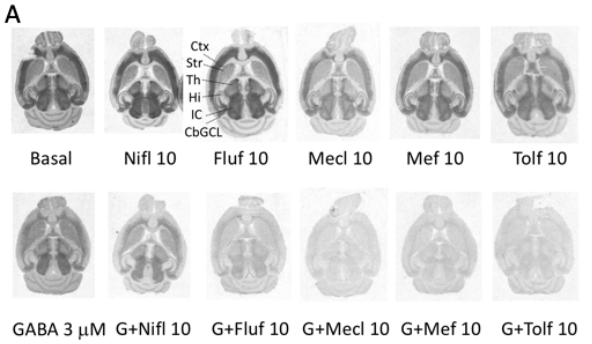
Fig. 6. (**A**) Representative current traces of niflumic acid and furosemide modulation on 30 μM and 20 μM GABA-induced currents in recombinant α1β1γ2 and α1β3γ2 $GABA_A$ receptors, respectively, expressed in *Xenopus laevis* oocytes. *n* = 7 and 5 for α1β1γ2 and α1β3γ2 combinations, respectively. F, furosemide 100 μM. (**B**) Niflumic acid effects represented as mean percentage ± S.E.M., the GABA 30 μM and GABA 20 μM control response being set to 100 %. F, furosemide 100 μM + GABA 30 μM or GABA 20 μM; N, 1000 μM niflumic acid alone. * $P < 0.05$, *** $P < 0.001$ for the significance of the difference from the corresponding control value (one-way ANOVA, followed by Dunnett's test for niflumic acid in the presence of GABA, Student's *t*-test for furosemide or niflumic acid alone). The dashed line represents the GABA control value. The EC_{50} values were 14.3 ± 3.8 μM and 33.0 ± 7.6 μM for α1β1γ2 and α1β3γ2, respectively.

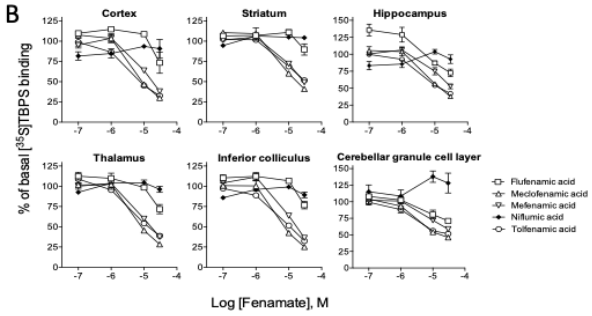
Fenamic acids and the related diclofenac

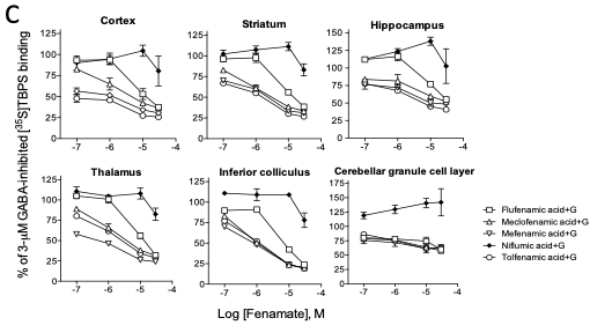


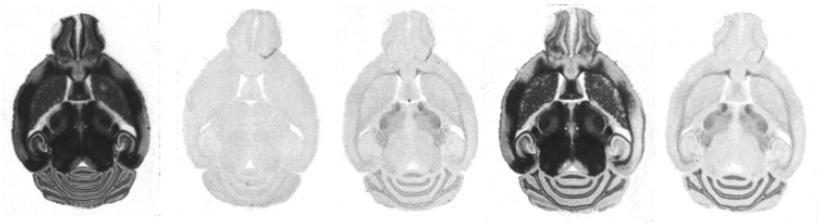
Other drugs used in the study



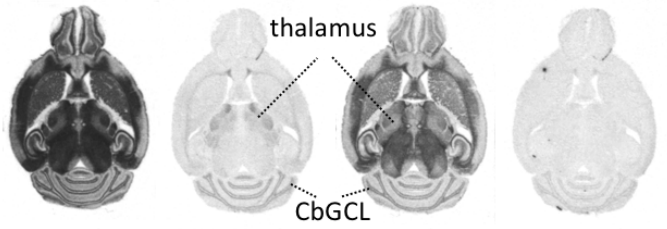

A
Ctx
Str
Th
Hi
IC
CbGCL
Basal
Nifl 10
Fluf 10
Mecl 10
Mef 10
Tolf 10
GABA 3 µM
G+Nifl 10
G+Fluf 10
G+Mecl 10
G+Mef 10
G+Tolf 10


B
Cortex
Striatum
Hippocampus
Thalamus
Inferior colliculus
Cerebellar granule cell layer
% of basal [35S]TBPS binding
Log [Fenamate], M
Flufenamic acid
Meclofenamic acid
Mefenamic acid
Niflumic acid
Tolfenamic acid

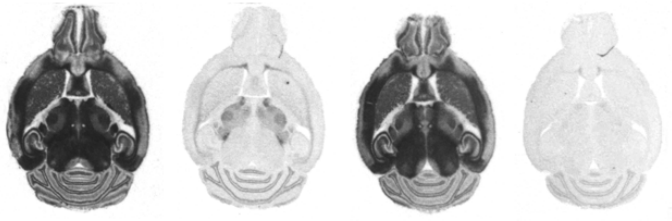

C
Cortex
Striatum
Hippocampus
Thalamus
Inferior colliculus
Cerebellar granule cell layer
% of 3-µM GABA-inhibited [35S]TBPS binding
Log [Fenamate], M
Flufenamic acid+G
Meclofenamic acid+G
Mefenamic acid+G
Niflumic acid+G
Tolfenamic acid+G



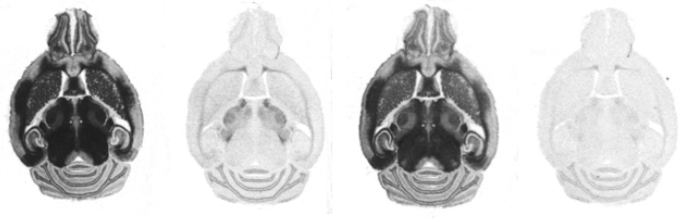
Basal Ptx, NS GABA 1 mM Nifl 100 G+Nifl 100



Mecl 1 G+Mecl 1 Mecl 10 G+Mecl 10

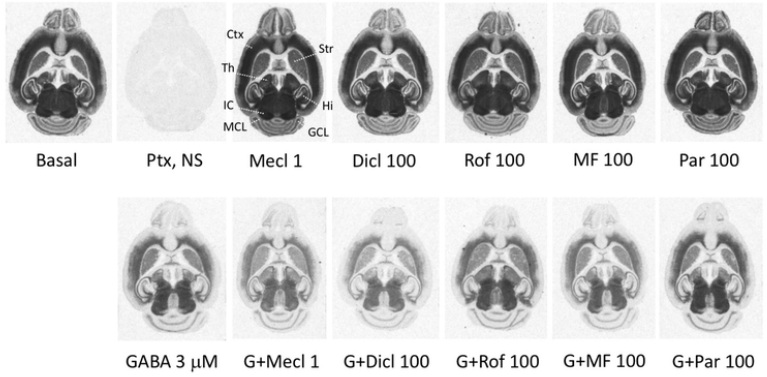


Mef 1 G+Mef 1 Mef 10 G+Mef 10

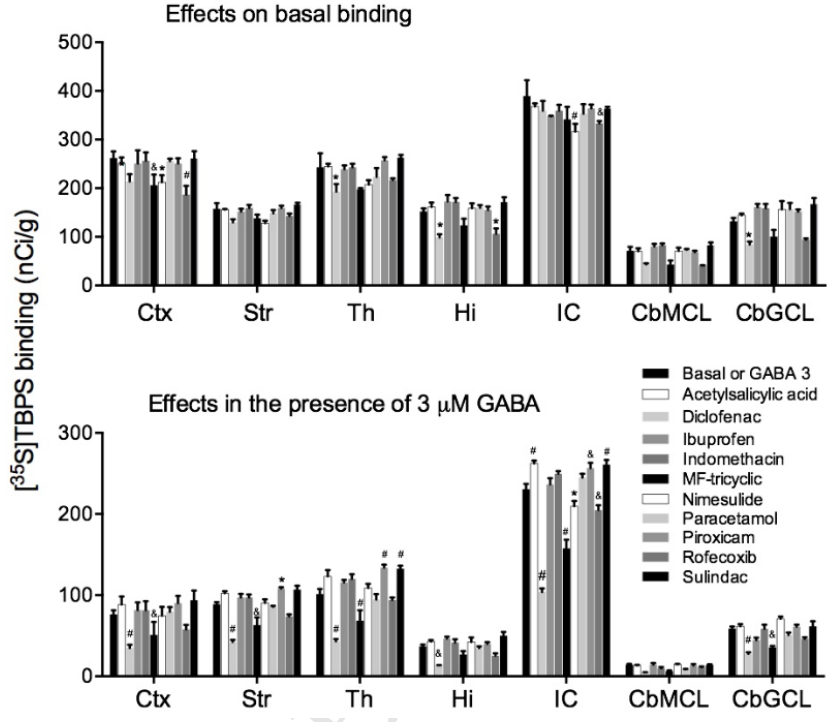


Tolf 1 G+Tolf 1 Tolf 10 G+Tolf 10

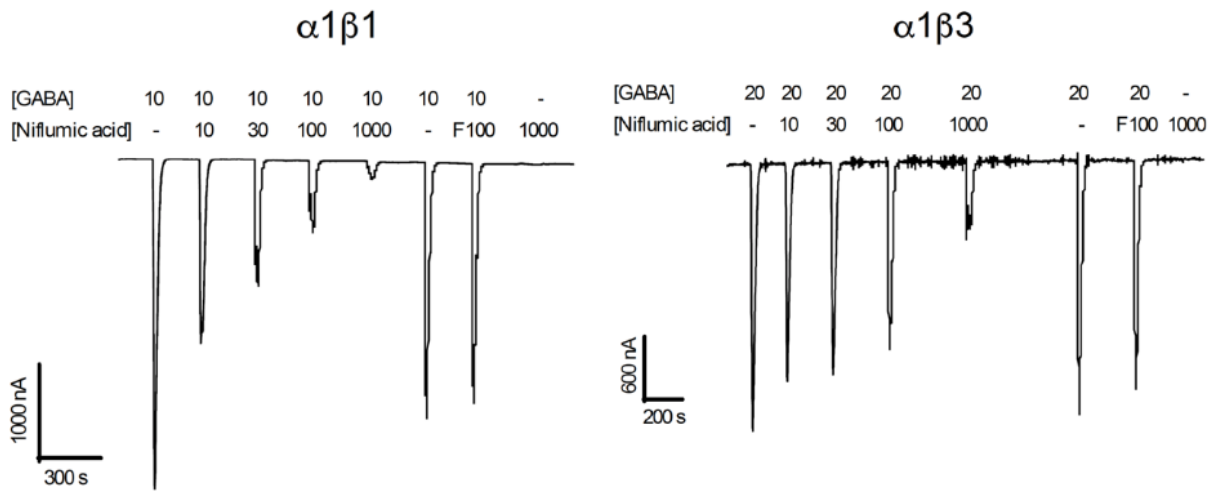
A


Ctx
Str
Th
IC
Hi
MCL
GCL
Basal
Ptx, NS
Mecl 1
Dicl 100
Rof 100
MF 100
Par 100
GABA 3 μM
G+Mecl 1
G+Dicl 100
G+Rof 100
G+MF 100
G+Par 100

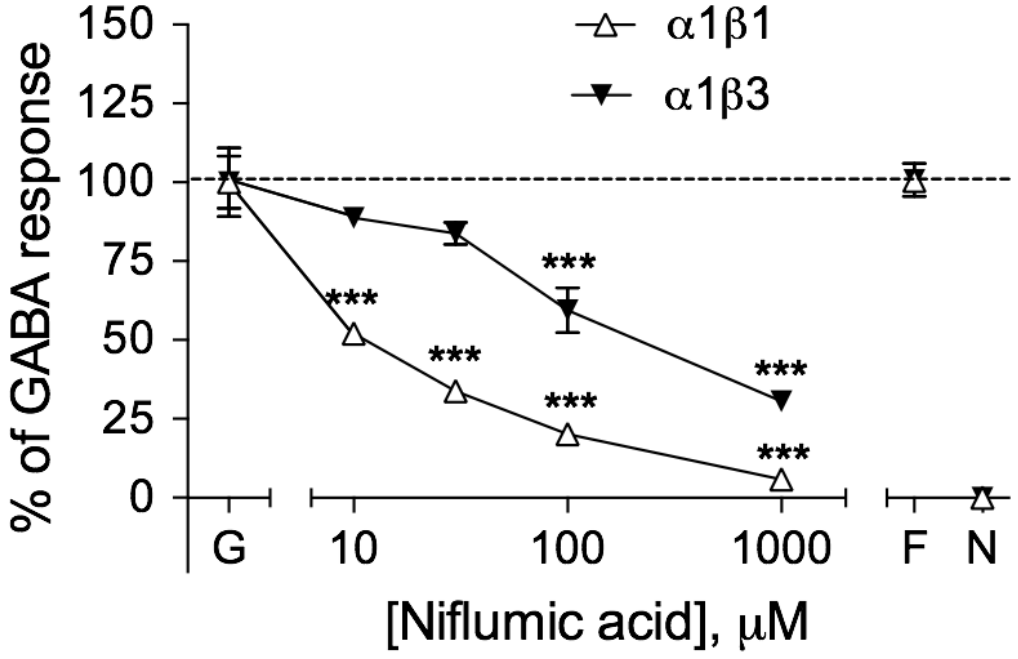
B


Effects on basal binding
[35S]TBPS binding (nCi/g)
500
400
300
200
100
0
Ctx
Str
Th
Hi
IC
CbMCL
CbGCL
Effects in the presence of 3 μM GABA
300
200
100
0
Basal or GABA 3
Acetylsalicylic acid
Diclofenac
Ibuprofen
Indomethacin
MF-tricyclic
Nimesulide
Paracetamol
Piroxicam
Rofecoxib
Sulindac

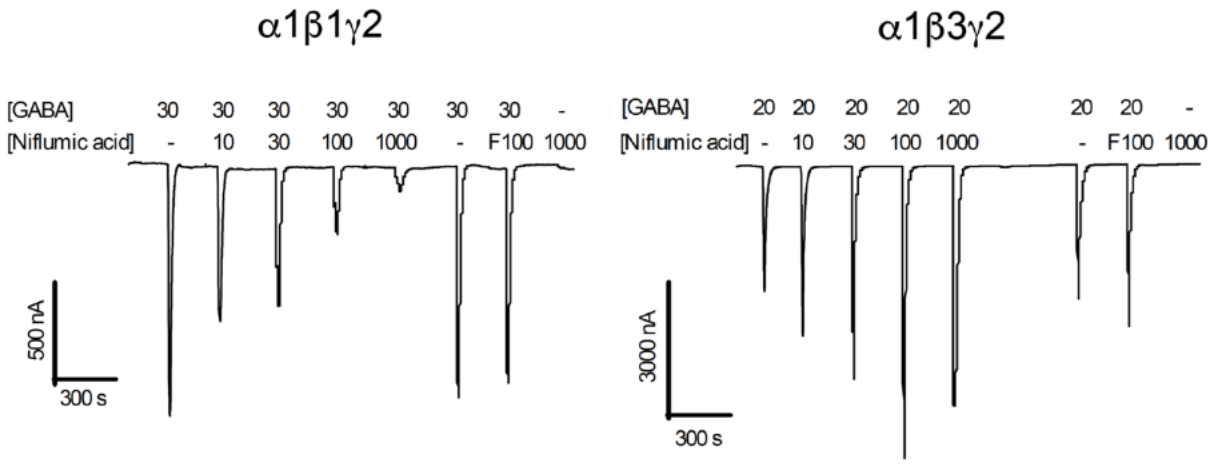
A


α1β1
α1β3
[GABA] 10 10 10 10 10 10 10 -
[Niflumic acid] - 10 30 100 1000 - F100 1000
[GABA] 20 20 20 20 20 20 20 -
[Niflumic acid] - 10 30 100 1000 - F100 1000
1000 nA
300 s
600 nA
200 s

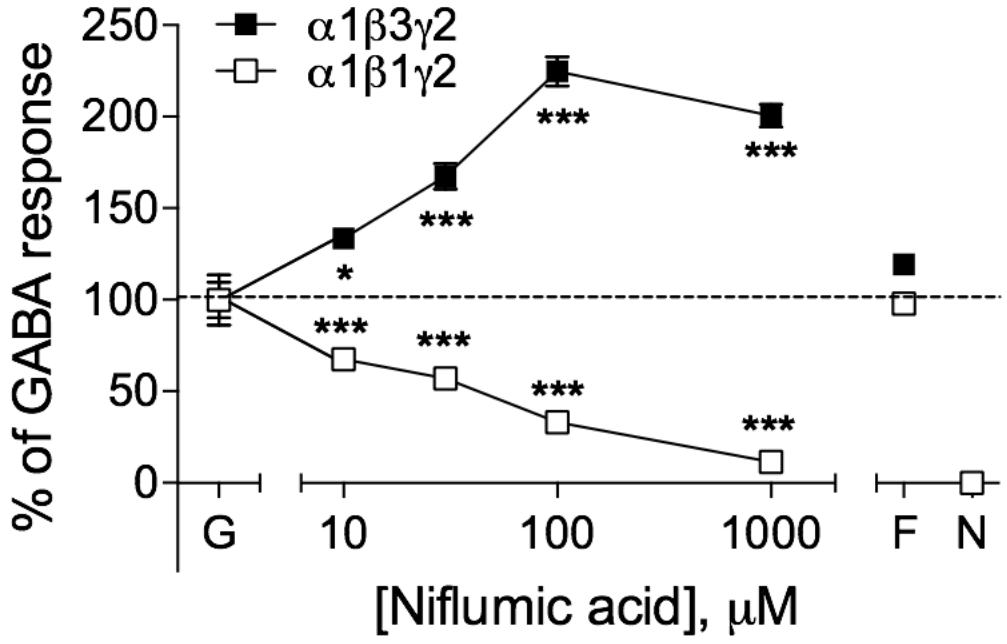
B


α1β1
α1β3
% of GABA response
150
125
100
75
50
25
0
***
G
10
100
1000
F
N
[Niflumic acid], μM

A


α1β1γ2
α1β3γ2
[GABA] 30 30 30 30 30 30 30 -
[Niflumic acid] - 10 30 100 1000 - F100 1000
500 nA
300 s
[GABA] 20 20 20 20 20 20 20 -
[Niflumic acid] - 10 30 100 1000 - F100 1000
3000 nA
300 s

B


α1β3γ2
α1β1γ2
% of GABA response
250
200
150
100
50
0
G
10
100
1000
F
N
[Niflumic acid], μM
*
***